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Multiregulatory Element of Filamentous Bacteriophages

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IG OF Ff BACTERIOPHAGES

Nestled in the 6,407-nucleotide, single-stranded deoxyribonucleic acid (DNA) ring (1, 2, 32, 83) of Ff phages (F-specific filamentious coliphages: f1, fd, M13, etc.) lies a 508-nucleotide, noncoding region (IG) (20, 82, 84). It is a multiregulatory element for the phage. As one moves from its 5′ end to its 3′ end, there is first a transcription termination signal for the 5′-proximal phage gene (63, 77), next a sequence required for virion morphogenesis (8, 14), then a sequence required for minus-strand synthesis (25, 39, 80), a further sequence required for plus-strand synthesis (39, 58, 79), and last, the promoter and operator for the 3′-proximal gene (16, 17, 66, 73).

It is these elements that largely control the life style of the phage. Uniquely among phages, these infective filaments can coexist with their host (33, 34, 45, 47, 51). The double-stranded replicative form is, in every way, analogous to a plasmid, and when it carries accessory genes, they can be expressed in the bacterial host (31, 52, 64, 65, 69). Normally, as single strands are synthesized, they are covered by a phage-encoded DNA-binding protein forming a structure not morphologically unlike the rod-shaped virion (67, 86). At the membrane of the cell, the binding protein is exchanged for the major coat protein and four phage-encoded minor proteins, to form the virion and be extruded into the medium without causing cell death (33, 34, 47). When the virion finds a bacterial host with a suitable pilus, infection proceeds and the cycle starts again.

ORGANIZATION OF GENES AND DNA

The Ff phages are plus-strand phages. All of their genes are transcribed in the same direction with the same polarity as that of the DNA in the virion. The phages have nine genes which code for 10 functional proteins (38, 46, 76) (see Fig. 1). Five genes encode virion proteins (VIII, III, VI, VII, and IX); two genes encode proteins involved in phage morphogenesis (I and IV); one gene codes a single-stranded DNA-binding protein (V); and the other gene encodes two proteins, one by the full length of gene II and the other, in the same reading frame, by its carboxy-terminal third (X). Gene II and X proteins are involved in the initiation and termination of plus-strand synthesis.

DNA replication of Ff phage occurs in three steps (57): (i) conversion of the infecting viral DNA into double-stranded replicative form (SS to RF); (ii) replication of the RF to produce a pool of RF molecules (RF to RF); and (iii) asymmetric synthesis of single-stranded viral DNA on the RF template (RF to SS). Only two mechanisms appear to be responsible for all three steps (19, 37, 39): synthesis of minus strands on a single-stranded plus-strand template to yield RF, and synthesis of plus strand which is initiated by gene II protein's nicking of the plus strand of RFI and which proceeds by elongation of the 3′ end of the nick with concomitant old plus-strand displacement. The products of one round of plus-strand synthesis are a circular single strand (SS) and a double-stranded circle (RF). Early in infection, the SS serves as template for minus-strand synthesis to yield more RF; later it is encapsidated into phage particles.

The intergenic region (IG) lies between the C terminus of gene IV and the N terminus of gene II (Fig. 1). Its 508 nucleotides are displayed in single-stranded form in Fig. 2. On the basis of potential loop-hairpin structures (71, 74, 75), the region is divided into several domains which provide the reference points for discussion of the various regulatory elements.

rho-Dependent Terminator in Region [A]

Because of the many *cis*-acting functions of the IG, it would probably be unwise for messenger ribonucleic acid (mRNA) transcription to enter it. Analysis of mRNAs synthesized after infection showed no RNA that covered the IG, indicating the existence of an mRNA terminator on its 5′ side (5, 43, 78). Various segments of the region were spliced into plasmid pKG1900. McKenney et al. (50) have developed a system for assaying termination based on inserting elements between a promoter and the galactokinase gene. The results of this assay, combined with the S1 mapping of phage mRNA from infected cells, led to the conclusion that mRNA is terminated on the distal side of the [A]-hairpin (position 5565), with small amounts proceeding to positions 5578 and 5650 (63). These latter two transcripts are seen in *rho*$^-$ hosts in addition to transcripts running 200 nucleotides further, although never through the IG. The amount of mRNA is severalfold larger in *rho*$^-$ and is probably due to the synthesis of more RNAs of different stabilities coming from a readthrough of upstream *rho*-dependent terminations (77). Because of the possibility of RNA processing, localization of a 3′ end does not prove termination. However, that messengers made in *rho*$^+$ and *rho*$^-$ strains have lengths consistent with the *rho*-dependent nature of the terminator,

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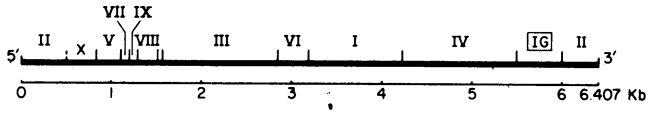

FIG. 1. Genetic map of Ff phage. The circular genome is presented in linear form as if it had been opened at the unique *Hin*dII restriction site. Roman numerals refer to the genes. IG is the intergenic region. X refers to the part of gene II that codes for the X protein. The direction of transcription and translation is from left to right. The bottom line represents scale marked in kilobases (kb).

when coupled with the results of the galactokinase system, provides strong evidence that position 5565 is the major site of termination. We note that, as for other *rho*-dependent terminations (70), there is only a loop-hairpin structure with no consensus nucleotide sequence.

Morphogenetic Signal

Viruses use various devices to package their DNA. For example, phages such as lambda have specific DNA cutting sites which allow their DNAs to fit the phage head (21), whereas other phages, whose DNA is also made in concatamers, have their DNA cut by the phage heads to fit (3, 81). Retroviruses have a specific sequence of identification for packaging (85), as do the Ff phages. Schaller (71) noted that a Tn*5* insertion into hairpin-[A] of fd markedly reduced its phage yield and guessed that this region was involved in morphogenesis. Insertion of segments of the IG into pBR (6, 8) were particularly revealing about morphogenesis and also led to the techniques that allowed much of the further analysis of IG function. Segments inserted into pBR included the whole IG ([A] through [F]) and regions [B] to [F], [B] to [E], [D] to [F], [D] to [E], [A] to [B], and [A] to [E] (Fig. 2). When *Escherichia coli* cells harboring these chimeric plasmids were superinfected with wild-type f1, three parameters were measured: yield of phage, yield of particles that transduce ampicillin resistance, and amount of plasmid DNA made (8).

The results led to the following picture of the functional arrangement of the IG. Whenever region [A] to [F] was present, transducing particles were formed. When [A] was absent, many fewer (about 1%) transducing particles were formed. Whenever [B] to [F] was present, plasmid RF increased in amount. Moreover, the yield of superinfecting phage was depressed 100-fold in comparison with cells containing plasmids lacking [B] to [F] (8). This is intepreted as follows. When there is a complete origin for DNA synthesis (see below), superinfection results in the chimeric plasmid entering an f1 mode of synthesis. Because there are many more copies of pBR per cell than superinfecting f1 DNA, their phage origins compete with those of f1 and the phage yield is reduced. In a complementary fashion, single strands formed from the competing plasmid are encapsidated and become ampicillin-transducing particles. (This phenomenon provides a useful method for obtaining single-stranded DNA of genes cloned into such chimeric plasmid vectors.) When the origin in the plasmid is incomplete, plasmid replication is not stimulated and a normal phage yield results. To package the particle, region [A] (morphogenetic signal) must be *cis* to the DNA element. The morphogenetic signal need not be contiguous with the IG to function (e.g., it can be inserted into another region of pBR), but it must retain the same 5′–3′ orientation relative to the origin that exists in the phage; the plus-strand sequence is what is recognized (8).

Further dissection of the hairpin reveals that, as a maximum, the region from nucleotide 5489 to 5563 is needed for packaging. These sequences encompass both sides of the hairpin-[A]. Either half alone does not function (14).

The precise role of the hairpin is yet to be defined. It is known, however, that its absence does not affect either single- or double-stranded DNA synthesis (14). In addition, a normal amount of gene V-DNA complex is found (27). What the morphogenetic signal may define is an end of the rod-shaped particles. The virion has been shown to have the products of genes VII and IX at one end and the products of gene III and VI at the other attachment end (26). By shearing in half wild-type particles and particles with a large DNA insert in the [A] to [B] region and collecting the gene III protein-carrying fragments, Webster et al. (87) have shown that the [A]-hairpin is probably opposite the gene III protein-containing end.

It should be noted, however, that some phage particles are made even in the absence of the [A] hairpin (6, 8). The hairpin evidently facilitates assembly but is not an absolute requirement. As it is still uncertain which elements of sequence or structure (or both) of the hairpin are required, it is possible that poorly functioning analogs exist elsewhere in the genome.

Minus-Strand Synthesis

The conversion of plus strands to double strands does not require any of the phage genes to function. DNA synthesis using host enzymes proceeds around the ring from a single position. It is initiated by a 30-nucleotide RNA primer (5756 to 5728) synthesized by the host's RNA polymerase (25).

The elements of structure that are required to initiate seem to be peaks [B] to [C]. In vitro RNA polymerase, in the presence of *E. coli* single-stranded DNA-binding protein, forms a complex with the phage plus strand and protects a specific region of the DNA from nuclease digestion (72). The protected region was shown to be some 125 nucleotides including the [B] and [C] hairpins (29). Deletion of [C] or

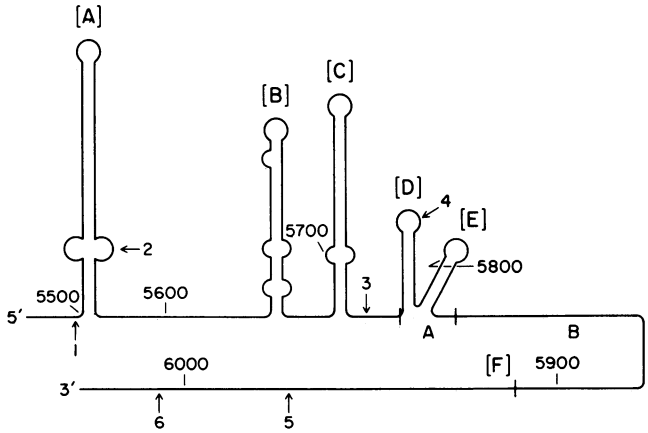

FIG. 2. IG region of Ff phage. Secondary structure of the viral strand DNA is schematically presented. [A], [B], [C], [D], and [E] represent self-complementary sequence elements. [F] is a ca. 150-base-long, AT-rich sequence without self-complementarity, extending downstream from the hairpin [E]. Shown by arrows are: (1) end of gene IV; (2) *rho*-dependent termination of gene IV mRNA; (3) initiation site of minus-strand primer synthesis; (4) initiation site of plus-strand synthesis; (5) initiation site of gene II mRNA; (6) start of gene II. Domains A and B of the plus-strand replication origin are indicated. Four-digit numerals represent nucleotide numbers.

insertion in [C] gives rise to phage that grow very poorly (41, 61, 62). In addition, defective interfering phage particles that evolve during phage passage often contain triplications or more of 50 nucleotides (5698 to 5748) of hairpin-[C] (36). These multimers possibly allow for a more efficient binding of RNA polymerase and could thereby account for the ability of these particles to outgrow wild-type phage.

As is true for the morphogenetic signal, the sequence about the minus-strand origin is not absolutely required. Its deletion results in phage which grow very poorly, producing <1% of the normal yield (41). The DNA sequences, if any, used in place of the deleted origin are unknown.

An f1 infection really begins with the synthesis of double-stranded DNA from the infecting single-stranded ring. The double-stranded ring is probably made superhelical by the cell's gyrase (37), thereby becoming a template for transcription and allowing for the messengers for the different phage gene products to be synthesized. Further synthesis of DNA requires gene II protein (gene X), while regulation of the ratio of single and double strands is accomplished by gene V protein (48, 49).

Plus-Strand Synthesis

Plus-strand synthesis involves the genome DNA around [D], [E], and parts of [F] and also the phage-encoded gene II, X, and V proteins. Gene V protein plays two roles: as the single strands are synthesized, it forms a complex with them in preparation for phage assembly at the cell membrane (28, 67, 86); it also negatively regulates the synthesis of gene II protein (59, 89) by binding to gene II mRNA around position 5980. Since the process of DNA synthesis begins by a nick introduced by gene II protein in the superhelical RF at position 5780 (24, 55, 68), regulation of the amount of gene II protein controls all of DNA synthesis and, ultimately, protein synthesis. Minus strands are only synthesized from free plus strands and messages from double-stranded DNA. It would seem, but is still uncertain, that it is the ratio of single strand to RF synthesis that allows Ff phages to grow in synchrony with their hosts.

An early result, that two phage origins could not be maintained in tandem in the phage (35), indicated that initiation and termination of plus-strand synthesis were probably intimately related. Since, in this day and age, one need scarcely leave a piece of DNA in its place to study it, phage origin(s) was inserted into plasmid pBR. The region about the plus-strand origin was subjected to in vitro mutagenesis (7, 11, 13). Deletions and insertions were produced. Mutants were identified by the chimera's inability to interfere with f1 superinfection, failure to produce transducing particles, and failure to reproduce its DNA by the f1 mode of replication. Mutations affecting initiation or termination were distinguished by a further test. When pBR contains two fully functional f1 origins about 800 bases apart in the same orientation, superinfection with f1 causes the chimera to break down into two rings, one large and one small, reflecting the spacing of f1 origins (10). A chimera containing a mutant origin and a wild-type origin yields only one ring. From knowledge of a mutation's position and orientation relative to the direction of DNA synthesis, it is easy to decide whether initiation or termination, or both, was affected by the mutation (12, 13).

One further assay is available, nicking of the mutant substrates by purified gene II protein. Gene II nicks between the T and A of TTAA at position (5780) (58). In the presence of Mg^{2+}, RFI is converted to equal amounts of RFII and RFIV; gene II protein is also a nicking-closing enzyme (11, 56). On the other hand, in the presence of Mn^{2+}, RFI is cleaved to RFIII (unit-length linear) (9, 56). These two activities both require superhelicity of the substrate.

By combining and analyzing all of the data obtained with the different mutants, the some 140 nucleotides of the plus-strand origin can be divided into two domains, A and B (Fig. 2). Domain B is the distal 100 nucleotides, rich in AT and dispensable to some extent (6, 7, 12, 13, 40). It is required only for plus-strand initiation (13). Superinfection of cells containing chimeras with deletions or insertions in this region gives a large yield of phage but only 1% of the normal number of transducing particles. In the initiation assay, these chimeras show only a low percent initiation. In vitro, the mutant DNAs are nicked by gene II protein, even at limiting concentrations (13).

The 40 nucleotides of domain A can in turn be divided into three partially overlapping sequences essential for viral strand replication: (i) a sequence required to catalyze termination, (ii) a sequence required for initiation, and (iii) a sequence required for in vitro nicking by gene II protein (13). Using the palindrome at [D] as a reference and calling the nicking site point "0," we find that termination requires nucleotides −12 to +(11–29); the complete palindrome [D] (12) perhaps is required to close the single-stranded rings as they are spun off (30, 54). The sequence required for initiation extends from nucleotide −4 to more than 100 nucleotides downstream. Therefore, excluding only the 5′ side of the [D] palindrome, it includes all of domains A and B and can be considered the phage functional origin.

The in vitro gene II nicking site extends from nucleotides −4 to nucleotides +(11–29) (11, 13). It thus includes the 5′ end of the initiation sequence as well as the 3′ end of the termination sequence. Since the initiation and termination sequences were determined by an in vivo assay, it is probable that the nicking sequence recognized in vitro is the one recognized in vivo. Moreover, it is most probable that this recognition sequence plays a major role in both the initiation and the termination of plus-strand synthesis. The 3′ end of domain A is not required for nicking but is necessary for a complete initiation event. This is in accord with the following results which indicate that gene II protein interacts with this region. When the intracellular level of gene II protein is low, the growth of superinfecting phage is strongly inhibited, even by plasmids containing certain defective f1 origins. A survey of the results with these deletion mutants shows that the sequence responsible for the inhibition is the 3′ end of domain A (around segment [E]), not the sequence required for the nicking (K. Horiuchi, manuscript in preparation). The 3′ end of domain A also has another phenotype. When it is inserted into a plasmid that has a functional f1 origin, it inhibits the f1 mode of replication by the plasmid.

These results suggest that, in addition to the nicking reaction, there is another type of interaction between gene II protein and the origin and that the region around the [E] palindrome is responsible for this interaction. Results of in vitro binding experiments of gene II protein to chimeric DNA are consistent with this notion (K. Horiuchi, unpublished data). It is probable that this interaction is related to the role of gene II protein in the formation, or initial movement, of the replication fork which follows the nicking event. It should be noted that in vitro synthesis of plus strands from purified RFII, made from RFI by gene II protein, still requires gene II protein (57).

When superinfected, chimeras carrying mutations in domain A allow a full yield of phage, of which <0.1% is transducing particles; the chimeras do not enter the f1 mode

of replication. They have completely defective origins whereas those with mutations in domain B are only partially defective. It appears that the sequences of domain B, like those of the morphogenetic signal and the minus-strand origin, are not absolutely necessary but, rather, facilitate plus-strand initiation (6, 7, 12, 13, 40).

When the coordinates of domain B were determined, a paradox developed. The Ff phages were used as vectors in recombinant DNA experiments (e.g., Messing's Mp series [52, 53]). The major site for insertion of foreign DNA was within domain B and yet the phages grew well (4, 90). However, newly formed and carefully cloned phages with such inserts in domain B grew poorly and formed an almost invisible plaque (15). Passage of such phage ultimately led to clear-plaque phages that grew quite well. Plating of the clear-plaque variants and Mp1 on chimeras carrying mutations in domain B, but not in domain A, led to superinfection interference, production of transducing particles, and stimulation of plasmid DNA synthesis—none of which obtains with wild-type phage (15, 16). This *trans*-acting effect made the plasmid's mutant origins act like a wild-type origin. When the replication functions were supplied by the Mp1 derivative, it was as if domain B had become superfluous to initiation (Horiuchi, unpublished data). These phage did not stimulate f1-specific DNA synthesis by domain A mutants.

Marker rescue experiments of these suppressor mutations revealed that one was a mutation in gene V, another was a mutation in the gene II leader, and Mp1 was mutated within gene II itself. The mutational changes are, respectively, as follows: an arginine-to-cysteine change at codon 16 of gene V (C to T) (15); a G-to-T change at position 5977 of the gene II leader sequence (16); and a methionine-to-isoleucine change at codon 40 of gene II (G to T) (16).

What ties these mutations together to give a common phenotype is their relationship to gene II function. In fact, their suppressor phenotype is nicely mimicked by growing mutant (domain B) phage on cells which contain a plasmid that hyperproduces gene II protein (15).

Gene V protein has been shown to control the translation of gene II mRNA (22, 59, 89). This implied that it is bound to a specific site (operator) on the messenger leader. The gene V mutation would then be a repressor mutation whereas that in the leader would be the cognate operator mutation. After infection, both lead to a five- to tenfold hyperproduction of gene II protein (15, 16). This increased concentration of gene II protein might well be the cause of their suppressor activity. The structural mutation in gene II does not lead to an increased concentration of gene II protein and hence its activity is more difficult to explain at present.

Gene II protein is involved in both initiation and termination of plus-strand synthesis. Its homolog in ϕX is covalently attached to the 5′ end of the plus strand (18, 44). This is not the case for gene II protein (56, 58). Initiation of DNA synthesis involves more than just the cleavage of a particular site. There must be some interaction with such cell proteins as *pol*III, *rep*, SS binding protein, etc. (57). We must remember that gene X protein (the C terminus of gene II) (42, 60, 88) is also a critical component for phage growth (23). Since wild-type gene II protein can cleave all domain B mutants, initiation of DNA replication of these mutants must be blocked at a higher level, possibly during the establishment of a growing fork. The role of the domain B nucleotides in this event is as yet obscure, but they do lie downstream from the beginning of synthesis. Possibly gene II protein interacts directly with domain B, but there is as yet no experimental evidence for this. Since both a mutant gene II protein and an increase in the concentration of the wild-type protein can render some 100 nucleotides totally dispensable, this represents a loosening of specificity rather than an acquisition of a new function.

CONCLUSION

The functional components of the intergenic space of the filamentous phages have been defined and precisely localized. However, except for the operator component for gene II mRNA regulation and the gene II cleavage site, there are few clues to how these DNA segments function. Some, such as that for the termination of gene IV mRNA and the initiation of plus-strand synthesis, involve double-stranded DNA; the others involve single-stranded DNA. Although domain B of the plus-strand origin can be said to be AT rich, as are many other origins of DNA synthesis, it is otherwise devoid of structural features, as is the rest of segment [F], which is also AT rich. The hairpins are probably important for the morphogenetic signal and the initiation of minus-strand synthesis, but obviously not any hairpin will do. Evidently we have a region in which there are sophisticated DNA-protein interactions about which we as yet know very little.

The structure of IG itself poses a mystery. The three *E. coli* Ff phages, f1, fd, and M13, all have an IG within one nucleotide of each other in length. Less than 5% of their nucleotide differ. Moreover, the regions between the regulatory signals can be deleted or contain insertions without obvious deleterious effect. Even the 12 nucleotides between the minus-strand origin and the plus-strand origin can have 11 nucleotides inserted into them without any effect on growth. Also, the virion filament adapts well to the DNA length it must contain. Why then are there nucleotide numbers conserved?

Locating many of the regulatory elements in one place, the IG, seems clever. Placing spacers between them seems wise. But maintaining such spacing when it seems not to have even a measuring function seems mysterious.

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